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14. ABSTRACT **Many now believe that the fallopian tube epithelium is the progenitor cell type for high-grade serous ovarian carcinoma. The initial phase of tubal cell metastasis could involve several sets of molecules, each of which represents a possible therapeutic target for intervention that would block serous cancer while still confined to the fallopian tubes. Using a series of normal, modified, and tumorigenic tubal cell lines, we will investigate the properties that allow tubal cells to migrate and adhere to novel three-dimensional ovarian organ cultures. We will also determine if the ovarian factors are necessary to fully transform fallopian tube cells. Using these as experimental models of pathway-modified or tumorigenic cells of tubal origin, we will investigate if ovarian factors enhance migration as a mechanism to explain the presence of tumor mass in the ovary of serous patients. 3D ovarian organ culture conditioned medium will be used as the chemoattractant. Collagen is a well-established matrix utilized by serous cancer cells, of unknown origin, to seed metastatic sites, such as the mesothelium. An RNAseq analysis will be performed between human TEC adhered to collagen matrix compared to tissue culture plastic and used to identify gene expression changes responsible for adhesion on collagen. Ovarian conditioned medium (OCM) with and without H2O2 treatment will be added to normal and our series of pathway-modified oviductal cell lines to determine if proliferation and growth in soft agar are enhanced by factors in the OCM**

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INTRODUCTION: The goal of this grant proposal is to understand how the ovary contributes to the migration, adhesion, and transformation of serous tumors that are derived from the fallopian tube. The initial metastatic step of HGSC appears to be spread of the fallopian tube epithelium (FTE) to the ovary. It is clear that the number of lifetime ovulation is correlated with the risk of developing ovarian cancer[1]. In mice, superovulation increases colonization of the ovary by cells injected into the uterine lumen[2]. These results suggest that ovulation contributes colonization of the ovary. Using ex vivo and in intro assay, we explored how and why FTE-cells adhere to the ovulatory wound of the ovary. Our results show that normal FTE attached the extracellular matrix (ECM) exposed during ovulation but the FTE must first become transformed before being able to growth in the collagen rich microenvironment. Intriguing, loss of a single gene (PTEN) activates multiple pathways that are important in ovarian colonization.

BODY: We have made significant progress and achieved the major deliverables for year 3 of our proposed research. **The first aim and major task 1 and 2** was to define ovarian secreted factors that enhance fallopian tube migration to the ovary, which was defined as activin and was published in *Cancer Letters*. After testing all of the cytokines with purified proteins, we found that none of them enhanced migration. Thus we proceeded to use a series of techniques that identified one of the key molecules as a protein called activin. We conducted experiments to block this with inhibin, follistatin, and a SMAD2DN construct. We also tested the importance of this in human cell lines. All of this is included in the manuscript in the appendix.

The second aim of this grant was to define the molecular requirements for tubal cells adhesion to collagen. We show that colonization of the ovary is a key metastatic step in progression of FTE-derived ovarian cancer. We also show that rupture of the ovary (as occurs during ovulation) increases the ability of FTE and HGSOC cells to attach the ovary due to exposure of the extracellular matrix (ECM). However, we found that normal FTE cells respond poorly to a 3D collagen rich microenvironment both by decreasing expression of genes involved with focal adhesion, regulation of the actin cytoskeleton, and ECM-receptor interactions, and by decreased viability. Testing several mutations (based on the RNAseq results), we found that loss of PTEN (but not activation of KRAS or mutation in p53) rescues the viability of FTE cells on 3D collagen, increasing their invasion through collagen, and increases attachment to the ovary. PTEN knockdown accomplished this by activating two different pathways (AKT and Rac1) that each contributes to the observed phenotypes. These data suggest that blocking colonization of the ovary may be therapeutic target in women at high risk of developing FTE-derived HGSOC and identified several potential targets.

FTE and HGSOC cells preferentially attached to the wound formed during ovulation.

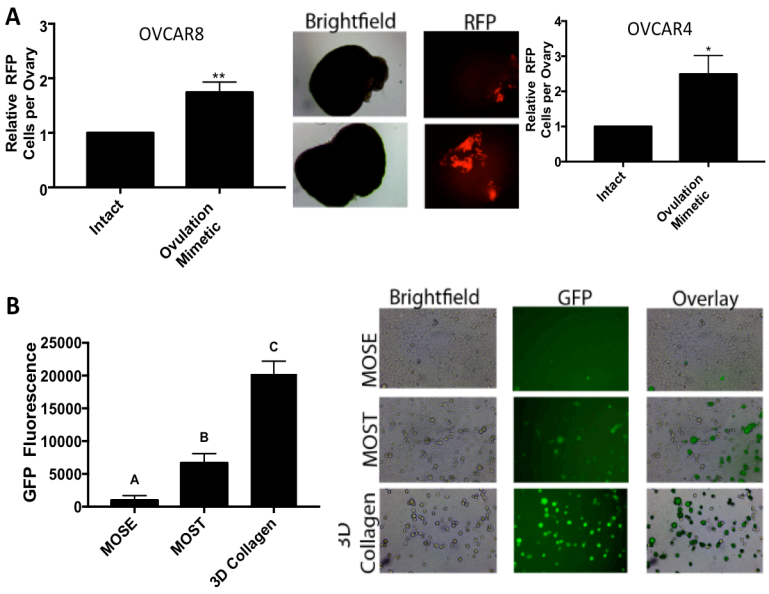

Figure 1. A) More OVCAR8 and OVCAR4 cells attach to ovulation mimetic ovaries more than intact ovaries. B) MOE GFP cells attached to 3D collagen > murine ovarian stroma (MOST)> murine ovarian surface epithelium (MOSE).

In our preliminary data for this grant, we showed that SKOV3 cells adhered to murine ovaries in areas devoid of OSE. However, SKOV3 are no long considered a good model of HGSOC. In year 2, we confirmed this observation using MOE (murine oviductal epithelial) cells tagged with GFP. In the last year, we have extended this observation, showing that cutting the ovarian surface (i.e. ovulation mimetic) increases the number of OVCAR4 and OVCAR8 cells (labeled with RFP) that attach to

murine ovaries (Figure 1). In year 2, we showed that MOE cells preferentially attached to 2D type I collagen rather than on top of MOSE cells, and as MOSE cell confluence increased, the attachment of MOE GFP cells decreased. To further validate our conclusion that FTE cells attached to the ECM exposed during ovulation, we added MOE GFP cells to a confluent monolayer of MOSE (to represent the intact ovary), a monolayer of primary murine ovarian stromal cell (MOST, to represent the ovulated ovary), and a 3D collagen gel (the primary ECM protein in the ovarian stroma). MOE GFP cells attached to MOST more than MOSE and to 3D collagen more than MOST (**Figure 1**), confirming that MOE cells adhere to the ECM exposed during ovulation. All of these data helped to improve upon the preliminary data that was presented in the grant application at the time of funding.

Transformation is required for FTE-cells to thrive in a collagen rich microenvironment.

Since the goal **of aim 2 and major task1** was to define the molecular requirements to attach to 3D collagen, in year 2 we performed RNAseq on MOE cells (Figure 2A and 2C). Counter to our expectations, KEGG analysis indicated the most down-regulated pathways included focal adhesion, regulation of actin cytoskeleton, and ECM-receptor interaction. These results suggest that normal FTE cells respond poorly to a 3D collagen rich microenvironment. We then validated some of these results with qPCR and western blotting as shown in Figure 3 to fulfill **Aim 2 major task 1**.

To fulfill **aim 2 major task 2**, we showed that 3D collagen reduced the viability of a panel of normal epithelial cells (MOE, MOSE, and

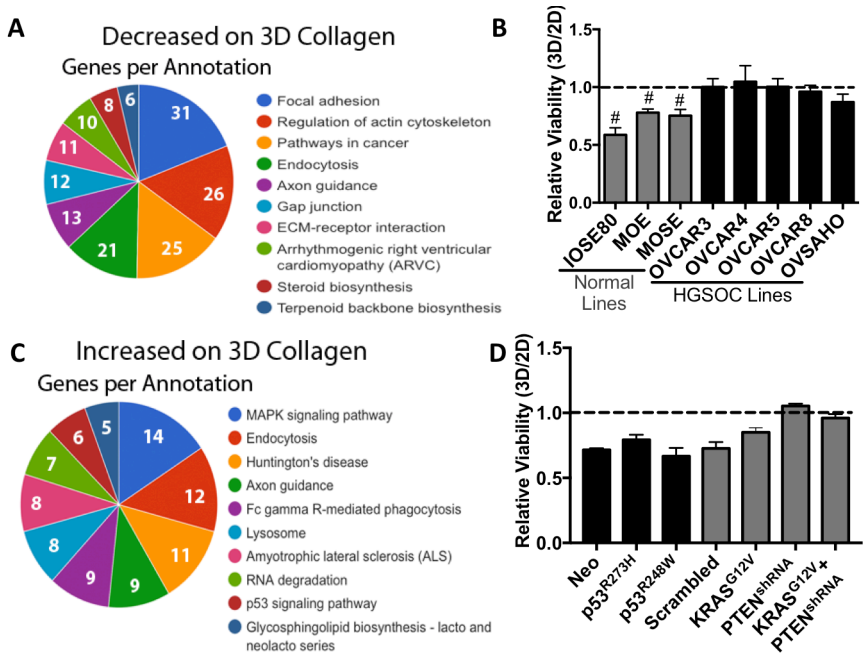

Figure 2. HGSOC and MOE PTEN^{shRNA} cells are unaffected by culture on 3D collagen.

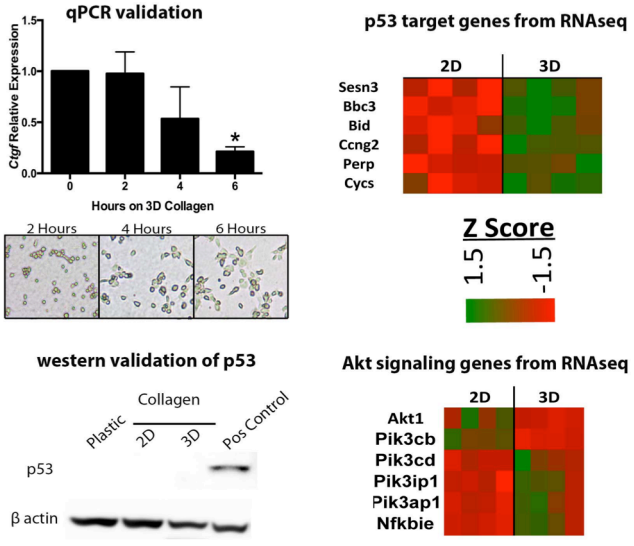

Figure 3. qPCR and western validation of p53 and Akt signaling from RNAseq.

IOSE80) but had no effect on several HGSOC cell lines (OVCAR3, OVCAR4, OVCAR5, OVAR8, and OVSAHO) (Figure 2B). These results suggest that normal FTE-cells must first be transformed prior to colonizing the ovary, which agrees with a recent publication shown that most mutations in HGSOC are acquired while still in the fallopian tube, before progression to the ovary[4]. This also supports our finding after year 1, where we identified that culture with the ovary did not enhance transformation, which was why we did not continue on with the original Aim 3 proposal. The RNAseq indicated significant increases in genes associated with p53 and MAPK/AKT signaling pathways (though the genes increased by 3D collagen and associated with MAPK/AKT are genes that would likely inhibit the pathway such as dual-specificity phosphatases, DUSP) (Figure 2A, 2C, and 3). Therefore, we analyzed the ability of mutant p53 (R273 and R248), activation mutation in KRAS ($KRAS^{G12V}$) and loss of PTEN (shRNA) to rescue the viability of MOE cells on 3D collagen. Neither mutation in p53 nor $KRAS^{G12V}$ was able to rescue the viability of MOE cells. In contrast, the viability of MOE $PTEN^{shRNA}$ was unaffected by 3D collagen (ratio of viability on 3D collagen:2D collagen=1; Figure 2D). These experiments also addressed **aim 2 major task 3**.

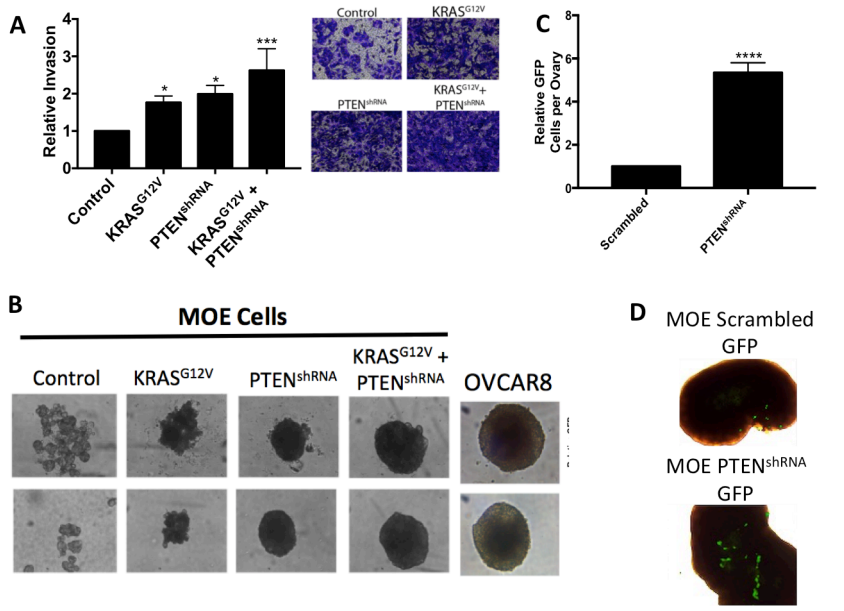

Figure 4. Loss of PTEN increases invasion (A), spheroid formation (B) and attached to ovaries (C and D).

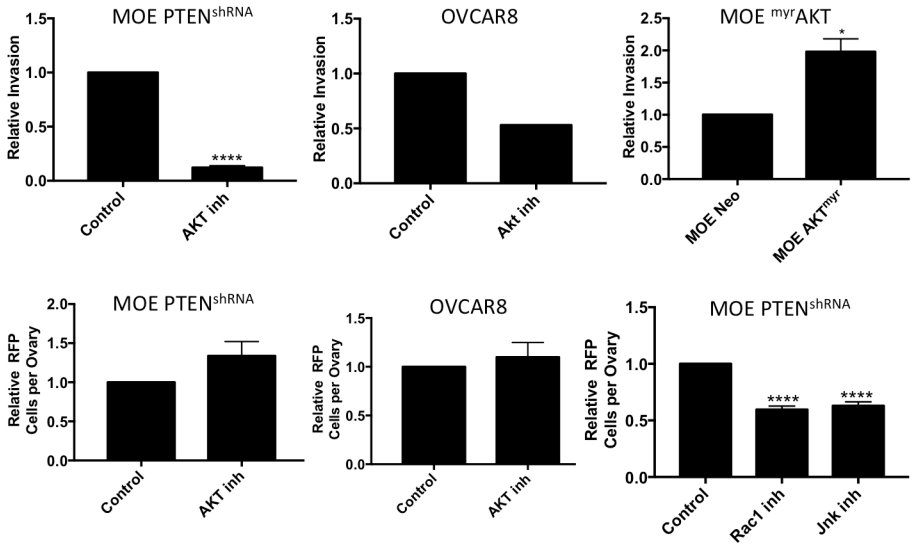

Figure 5. AKT mediated increased invasion of MOE $PTEN^{shRNA}$ cells but Rac1/JNK mediate increased attachment.

Loss of PTEN increase the ability of FTE-cells to attached to ovaries, invaded through collagen, and form spheroids through AKT and RAC1.

We next investigated what other phenotypes loss of PTEN might confer in relation to 3D collagen. Loss of PTEN (and $KRAS^{G12V}$) increased invasion through collagen. $PTEN^{shRNA}$ also allowed the MOE cells to form spheroids after 7 days on 3D collagen. Using MOE Scrambled GFP and MOE $PTEN^{shRNA}$ GFP cells, we also show that PTEN increased adhesion to ovulation mimetic ovaries (Figure 4).

Loss of PTEN is well known to activate AKT signaling, however, it also activates Rac1/JNK signaling. Therefore, we tested the role of these two pathways in the increased viability, attachment, invasion, and spheroid formation of MOE $PTEN^{shRNA}$ cells. The AKT inhibitor MK2206 dramatically reduced invasion of both MOE $PTEN^{shRNA}$ and OVCAR8 cells through collagen, suggesting that activation of AKT is a key mediator of increased invasion. To confirm this, we tested the ability of myristoylated AKT (AKT^{myr}) to increase invasion as myristoylation increases activation of AKT without increase levels of PIP3, which activate Rac1. AKT^{myr} doubled the invasion of MOE cells through collagen, which was the same magnitude seen in $PTEN^{shRNA}$ cells, confirming that activation of AKT is responsible for the increased invasion (Figure 5).

In contrast, MK2206 had no effect on MOE $PTEN^{shRNA}$ RFP cells or OVCAR8 RFP cells to attach to ovaries, suggesting this effect is independent of AKT. However, both the Rac1 inhibitor and JNK inhibitor reduced attachment by 40%. These results indicate that AKT and RAC1/JNK mediate different aspects of the phenotypes seen in MOE $PTEN^{shRNA}$ cells.

Spheroids may be the metastatic unit from the fallopian to the ovary.

The observation that MOE $PTEN^{shRNA}$ cells form spheroids was intriguing. Spheroids are thought to mediate metastasis from the ovary to the peritoneum. These data lead us to hypothesize that spheroids may also mediate metastasis from the fallopian tube to the ovary. To determine if spheroids could attach to ovaries wounded to mimic ovulation, we incubated spheroids produced from OVCAR8 RFP cells with murine ovaries (intact or ovulation mimetic). Ovulation mimetic significantly increased the percentage of ovaries with spheroids attached (Figure 6).

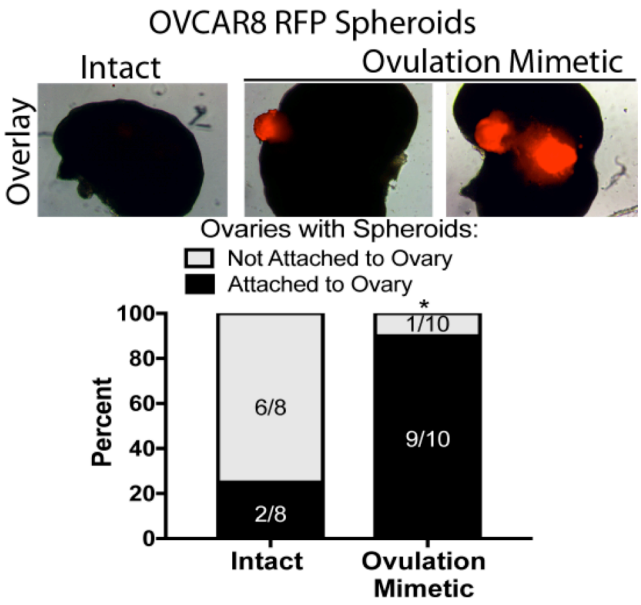

Figure 6. OVCAR8 RFP spheroids attach to the wounded surface of murine ovaries.

As was mentioned in the previous progress reports, the ovarian microenvironment was not able to transform fallopian tube cells and was not pursued for **aim 3**.

Progress of TEAL Postdoc: Matthew Dean was added to this grant to replace Sharon Eddie. During this time, he was the first author on a Cancer Letters paper and is currently preparing a manuscript with the data from this report. He was recently named an Ann Schreiber Fellow recipient from the Ovarian Cancer Research Fund that will support him from 2018-2020.

KEY RESEARCH ACCOMPLISHMENTS:

- Colonization of the ovaries makes fallopian tube epithelium (FTE)-derived tumorigenic cells much more aggressive.
- FTE cells preferentially bind to ECM proteins that are exposed during ovulation.
- Normal FTE cells responds poorly to a 3D collagen rich microenvironment, but the viability of HGSOC and FTE cells that lack PTEN is unaffected.
- FTE cells that lack PTEN also invade through collagen and attach to ovaries more than control FTE cells, and FTE cells without PTEN form spheroids after 7 days in 3D collagen.
- Both AKT and Rac1 mediate the effects of $PTEN^{shRNA}$.
- Spheroids may mediate metastasis from the fallopian tube to the ovary.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- **Abstracts and presentations**

Dean M, Vivian J, Davis DA, and Burdette JE. (2017) The role of the ovary in fallopian tube derived ovarian cancer. The Endocrine Society Annual Meeting, Orlando FL.
*Received Outstanding Abstract Award.
*Selected for oral Knockout Round Presentation
*Won Presidential Poster Competition in Tumor Biology

Dean M, Vivian J, and Burdette JE. (2016-2017) Ovarian colonization of fallopian tube epithelium-derived high-grade serous tumors: an important step in metastasis.
Women's Health Research Day. University of Illinois at Chicago. Chicago, IL.
College of Pharmacy Research Day. University of Illinois at Chicago. Chicago, IL.
*1st Place Poster in Biology Category.
Cancer Center Research Forum. University of Illinois at Chicago. Chicago, IL.

Dean M, Vivian J, Russo A, and Burdette JE. (2017) PTEN and colonization of the ovary in metastasis of fallopian tube derived ovarian cancer. AACR Addressing Critical Questions in Ovarian Cancer Research and Treatment. Pittsburg, PA.

- **Publications**

Dean M, Jin V, Russo A, and Burdette JE. (2017) Colonization of the Ovary During Ovulation Loss of PTEN are Important Drivers of Fallopian Tube-Derived Cancer Metastasis. In preparation.

Dean M, Davis DA, and Burdette. (2017) Activin A stimulates migration of the fallopian tube, epithelium an origin of high-grade serous ovarian cancer, through non-canonical signaling. Cancer Letters. 391: 114-124. PMID: 28115208

- **degrees obtained that are supported by this award**
- **development of cell lines, tissue or serum repositories**
- **funding applied for based on work supported by this award**

The ovarian microenvironment in metastasis of fallopian tube derived cancer. (awarded March 2018- 2020) Ann Schreiber Mentored Investigator Award. Ovarian Cancer Research Fund Alliance. PI Matthew Dean

The role of the ovary in high grade serous cancer formation (pending). R01 NIH PI Burdette

CONCLUSION: Our results show that colonization of the ovary is a key step of metastasis of FTE-derived tumors to the peritoneum. Our previously work identified activin A as a chemotactic protein produced by the ovarian follicles that simulate migration of FTE and HGSOC cells. Now we show that FTE and HGSOC cells attach to the wound produced during ovulation. However, normal cells respond to a 3D collagen rich microenvironment. We found that loss of PTEN resulting in increased attachment and invasion to the ovary. Loss of PTNE also allowed FTE cells to form spheroids. These effects of PTEN loss was during activation of at least two distinct pathways: AKT and RAC1/JNK. This work increases our understanding of the role of the ovary in fallopian tube derived cancer and highlights pathways that contribute to the tumorigenic of these cells.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

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APPENDICES: Dean M, Davis DA, and Burdette. (2017) Activin A stimulates migration of the fallopian tube, epithelium an origin of high-grade serous ovarian cancer, through non-canonical signaling. Cancer Letters. 391: 114-124. PMID: 28115208

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Original Articles

Activin A stimulates migration of the fallopian tube epithelium, an origin of high-grade serous ovarian cancer, through non-canonical signaling

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ABSTRACT

Factors that stimulate the migration of fallopian tube epithelial (FTE)-derived high-grade serous ovarian cancer (HGSOC) to the ovary are poorly elucidated. This study characterized the effect of the ovarian hormone, activin A, on normal FTE and HGSOC. Activin A and TGFβ1 induced an epithelial-to-mesenchymal transition in murine oviductal epithelial (MOE) cells, but only activin A increased migration. The migratory effect of activin A was independent of Smad2/3 and required phospho-AKT, phospho-ERK, and Rac1. Exogenous activin A stimulated migration of the HGSOC cell line OVCAR3 through a similar mechanism. Activin A signaling inhibitors, SB431542 and follistatin, reduced migration in OVCAR4 cells, which expressed activin A subunits (encoded by INHBA). Murine superovulation increased phospho-Smad2/3 immunostaining in the FTE. In Oncomine, transcripts for the activin A receptors (*ACVR1B* and *ACVR2A*) were higher in serous tumors relative to the normal ovary, while inhibitors of activin A signaling (*INHA* and *TGFB3*) were lower. High expression of both *INHBA* and *ACVR2A*, but not TGFβ receptors or co-receptors, was associated with shorter disease-free survival in serous cancer patients. These results suggest activin A stimulates migration of FTE-derived tumors to the ovary.

Introduction

Ovarian cancer is the fifth leading cause of cancer death in women and the most lethal gynecologic malignancy [1]. Epithelial ovarian cancers were historically thought to arise from the ovarian surface epithelium (OSE). However, it is now clear that high-grade serous ovarian cancer (HGSOC), the most common and deadly histotype, can also arise from the fallopian tube epithelium (FTE, analogous to oviductal epithelium in non-primate species). As evidence of an FTE origin, several studies identified putative precursor lesions in the fallopian tube of women undergoing prophylactic oophorectomy called p53 signatures, which were patches of secretory epithelium that express mutant p53 protein [2–4]. The transcriptomic profile of HGSOC tumors significantly correlated with that of the fallopian tube [5], and the genomic methylation patterns of HGSOC were more similar to the FTE than the OSE [6]. A recent retrospective analysis found salpingectomy was associated with a 45% decrease in ovarian cancer risk [7]. Our laboratory has shown that murine oviductal epithelial (MOE) cells stably expressing $PTEN^{shRNA}$, $PTEN^{shRNA}$ + $KRAS^{G12V}$, or $PTEN^{shRNA}$ + $TP53^{R273H}$ colonized the ovary following intraperitoneal xenograft [8,9]. Several transgenic mouse models have also been developed that form fallopian tube-derived tumors that spread to the ovary [10–12].

Factors other than physical proximity likely contribute to colonization of the ovary by fallopian tube-derived tumors. For example, Coffman et al. found that when injected intra-venously, HGSOC cells, but not breast cancer or lung adenocarcinoma cells, colonized the ovary [13]. The lifetime number of ovulations is positively associated with the risk of developing ovarian cancer [14]. Yang-Hartwich et al. found that superovulation, following xenografting of tumorigenic cells into the uterine lumen, increased the proportion of mice with ovarian tumors [15]. In a transgenic mouse model of fallopian tube-derived cancer, ovariectomy reduced peritoneal metastasis [10]. These data suggest that HGSOC cells preferentially colonize the ovary, a phenomena linked to

Abbreviations: FTE, fallopian tube epithelium; HGSOC, high grade serous ovarian cancer.

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0304-3835/

ovulation, and indicate that blocking this migratory step may represent a therapeutic target in women. However, the factor(s) that draw HGSOC cells to the ovary are just beginning to be elucidated [15].

Activin A is a member of the TGFβ superfamily and a classic ovarian hormone. Activin A is composed of two inhibin βA subunits (gene symbol *INHBA*), while a heterodimer of inhibin α (gene symbol *INHA*) and inhibin βA produce inhibin A. Activin A signals by binding the type I and type II activin receptors (gene symbols *ACVR1B* and *ACVR2A,* respectively). Classically, activin A and TGFβ receptors phosphorylate Smad2/3; however, both hormones can activate other signaling pathways, such as PI3K/AKT and ERK/MEK, in a ligand and cell dependent manner. Inhibin A blocks activin signaling at the receptor level, and follistatin binds directly to activin A by sequestering it away from the receptor [16].

Activin A and TGFβ have been shown to stimulate migration and invasion of various cell types through activation of PI3K/AKT and MEK/MAPK signaling [17]. These pathways can be activated either directly or indirectly through the actions of phospho-Smad2/3. Ligand binding causes the TGFBRII receptor to interact directly with growth factor receptor-bound protein 2 (Grb2) and Shc-tranforming protein (Shc), leading to MEK/MAPK signaling [18]. Both TGFβ and activin A have been shown to activate transforming growth factor beta-activated kinase 1 (TAK1) and increase MEK/MAPK signaling [19,20]. There is also a direct interaction between TGFBRI and the p85 subunit of PI3K, leading to ligand-induced phosphorylation of AKT [21,22]. In addition, the type II activin receptor coimmunoprecipitates with p85 [23]. Activin A and TGFβ activate Rho GTPases (RhoA, Rac1, and Cdc42), which are important regulators of migration. For example, RhoA mediated activin A-induced migration in keratinocytes [24], while TGFβ stimulated migration of pancreatic ductal adenocarcinoma through Rac1 [25]. However, the specific Rho GTPase required for migration of the FTE is unknown.

Increased levels of activin A have been linked to several cancer types. For example, activin A serum concentrations are associated with tumor progression in lung cancer [26]. Additionally, activin A is overexpressed in colorectal cancer [27]. In breast cancer, activin A induces an epithelial-to-mesenchymal transition (EMT) and stimulates migration [28]. In the normal ovary, activin A plays a role in the growth of follicles and their steroidogenesis [29], with concentrations of 5–50 ng/ml of activin A being measured in follicular fluid [30]. In ovarian cancer, activin A is well known to stimulate development of stromal cell tumors, as evidenced by development of these tumors in *INHA* knockout mice [31]. The role of activin A in epithelial ovarian cancer is less clear. Basu et al. observed higher phospho-Smad2 immunostaining in ovarian tumors compared to normal ovaries [32], and Do et al. found that ovarian cystadenocarinoma patients with high immunostaining for INHBA tended to have shorter survival times than patients with low immunostaining [33]. Activin A stimulates migration of OCC1 and SKOV3 cells [34]. However, OCC1 cells were isolated form ovarian clear cell carcinoma, and recent genomic analysis indicated that SKOV3 cells are not representative of HGSOC [35,36].

The objectives of the current study were to 1) characterize the effects of ovarian-produced activin A on migration and proliferation of the FTE, 2) elucidate the pathways by which activin A stimulates migration of the FTE and determine if it is conserved in HGSOC cell models, and 3) explore expression of activin A (i.e. INHBA) and activin receptors (*ACVR1B* and *ACVR2A*) in HGSOC patient tumors.

Materials and methods

Cell culture

Spontaneously immortalized murine oviductal epithelial (MOE) cells were a gift from Barbara Vanderhyden at the University of Ottawa and had previously been used by our laboratory to model the FTE [8,9]. MOE cells were maintained in αMEM (10-022-CV, Cellgro, Manassas, VA) supplemented with 10% FBS (FB5001, Denville Scientific, Holliston, MA), 2 mM L-glutamine (30005068, CellGro, Manassas, VA), 10 μg/ml ITS (11074547001, Roche, Indianapolis, IN), 1.8 ng/ml EGF (100-15, Peprotech Inc, Rocky Hill, NJ), 100 U/ml penicillin-streptomycin (15140-122, Gibco, Grand Island, NY), 1 μg/ml gentamycin (30-005-CR, GellGro, Manassas, VA), and 18.2 ng/ml estradiol-17β (E1024-1G, Sigma Aldrich, St. Louis, MO). OVCAR4 cells were maintained in RPMI 1640 media (11875-093, Life Technologies, Waltham, MA) supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine. OVCAR3 cells were maintained in MEM supplemented with 20% FBS, 1% non-essential amino acids (11140050, Gibco, Grand Island, NY), 1% L-glutamine, 0.1 mg/ml insulin (700112P, Gemini Bioproduct, West Sacramento, CA), and 100 U/ml penicillin-streptomycin. All cells were maintained in T-25 or T-75 flasks at 5% CO_2 and 37 °C in a humidified incubator. Each cell line had been authenticated by STR analysis within the past 2 years. Cells were collected with 1× trypsin/EDTA (15400-054, Life Technologies, Waltham, MA) and passed at confluence.

Hormones, inhibitors, and RNAi

Activin A and TGFβ1 were purchased from R&D Systems (338-AC-010 and 7666-MB-005, respectively; Minneapolis, MN). TGFβ1 was dissolved in 4 mM HCl at 10 mg/ml, and activin A was dissolved in 4 mM HCl with 0.1% BSA at 10 mg/ml. Follistatin 228 (a gift from Teresa Woodruff at Northwestern University) was dissolved in PBS and used at 200 ng/ml. Rhosin was purchased from Fisher Scientific (5003, Waltham, MA). All other inhibitors (MK2206, 11593; U0126, 70970; MLS573151, 15369; SB431542, 13031; and NSC23766, 13196) were purchased from Cayman Chemical (Ann Arbor, MI). All inhibitors were dissolved in DMSO at 1,000× and added to media prior to adding treatments to each well.

The Smad2-dominant negative (Smad2DN) construct was a gift from Teresa Woodruff at Northwestern University and has been previously validated [37,38]. Cells were transfected with 500 ng/ml Smad2DN plasmid or empty vector (pcDNA3) with ViaFect (E4981, Promega, Madison, WI) following the manufacturer's instructions. Luciferase^{siRNA} (Luc^{siRNA}) and Rac1^{siRNA} were purchased from Sigma (EHURLUC-50UG and EMU028841-20UG, respectively; St. Louis, MO). Each well was transfected with 25 nM siRNA using TransIT-X2 (MIR 6004, Mirus, Madison, WI) following the manufacturer's instructions. The cells were incubated 24 h post-transfection before performing experiments.

Western blotting

Cells (150,000–450,000 per well, depending on cell line) were plated in 6-well plates in complete media. The next day the media was replaced with serum free MEM. Twenty-four hours later, media with appropriate treatments were added to each well and cell lysates were collected at indicated times in RIPA buffer containing protease and phosphatase inhibitors [39]. Protein concentrations were determined with a BCA assay (23227, Thermo Scientific, Waltham, MA), and 25 μg of protein was separated in a 10% SDS-PAGE gel by applying constant voltage (100 V) for 120 min. Proteins were transferred to nitrocellulose membranes, blocked for 1 h, and incubated overnight in primary antibody. The next day the membrane was washed three times in TBS-T, incubated with appropriate secondary antibody (anti-rabbit 1:1000 or anti-donkey 1:10,000) conjugated to horseradish peroxidase for 30 min at room temperature. Membranes were then washed three times with TBS-T, and developed using SuperSignal West Femto Maximum Sensitivity Substrate (34095, Thermo Scientific, Waltham, MA). Images were captured with a FluorChem E (ProteinSimple, San Jose, CA). The primary antibodies, dilutions, and blocking conditions are listed in Table S1.

Migration assays

For scratch assays, a monolayer of cells was seeded onto 24-well plates. After ~ 6 h later (OVCAR3) or the next day (MOE and OVCAR4 cells) a uniform scratch was made down the center of each monolayer with a 1000 μl pipette tip. OVCAR3 cells were cultured overnight to achieve confluence (minus the scratched area) before treatment. After scratching, the monolayer was washed two times with PBS and serum free MEM with appropriate treatment was added to each well. Images were captured at 4× at 0 and 24 h (MOE and OVCAR4) or 0 and 48 h (OVCAR3). The area of each scratch within the field of view was determined with ImageJ software (http://imagej.nih.gov/ij/).

For Boyden chamber assays, 0.5 ml per well of MEM with appropriate treatments was added to 24 well plates. MOE cells were collected from a T-75 flask with trypsin/EDTA and MOE media. Cells were washed two times with FBS-free MEM and 100,000 MOE cells were added to each insert (8.0 μM pores; PI8P01250, Millipore, Billerica, MA) free of any extracellular matrix proteins and placed in each well. Plates were incubated for 6 h, and cells that had not migrated through the insert were removed with a cotton swab. Cells on the bottom of the insert were fixed with 4% PFA, permeabilized with 70% methanol, and stained with 0.2% crystal violet. Each insert was washed two times with PBS and dried. Images were captured at 4× to count the number of migrated cells. Cell number was normalized to control.

SRB proliferation assay

Cell proliferation was determined using the sulforhodamine B (SRB) assay [40]. Five hundred MOE cells per well were plated in 100 μl of MEM in 96-well plates. Two hours later the first plate (day 0) was collected and indicated treatment was applied to a second plate, which was collected at day 5. At collection, media was decanted, cells were fixed with 100 μl 20% trichloracetic acid (TCA), and stored at 4 °C until development. Plates were rinsed four times with tap water and then 100 μl of SRB was added to each well and incubated for 30 min at room temperature. Plates were then washed four times with 1% acetic acid, dried, and SRB was resuspended with 200 μl of Tris buffer. Absorbance was measured at 505 nm using a Synergy 2 microplate reader (BioTek, Winooski, VT). Values are expressed as fold increase over day 0.

Murine superovulation

All animals were treated in accordance with the National Institute of Health Guide for the Care and use of Laboratory Animals. Animals were housed in a temperature and light (12L:12D) controlled environment. Water and food were provided *ad libitum*. CD-1 mice were obtained from in-house breeding, and superovulation was induced as previously described [41]. Briefly, mice were treated on days 1 and 2 with PBS on both days (control) or 5 IU PMSG (day 1) followed by 5 IU hCG on day 2 (superovulation). Twelve hours after the second injection mice were sacrificed, the reproductive tract was excised and fixed in 4% PFA.

Immunohistochemical staining for phospho-Smad2/3 was carried out as previously described [38]. Reproductive tracts were fixed in 4% PFA, embedded in paraffin, and sectioned at 5 μm. Sections were probed with primary antibody against phospho-Smad2/3 (Table S1) overnight, washed, and probed with secondary antibody (1:200 biotinylated anti-rabbit; BA-1000, Vector Laboratory, Burlingame, CA). After washing, slides were mounted and images were captured at 20× or 100× with a Nikon Eclipse E600 microscope.

Oncomine™ analysis

Oncomine™ (v4.50, IonTorrent, http://https://www.oncomine.org/resource/login.htmlhttp://https//www.oncomine.org/resource/login.html) is a privately curated database of microarray and sequencing experiments specific to cancer biology. Oncomine™ was explored and 4 studies of interest were identified [42–45] that compared gene expression in serous ovarian tumors to normal tissue. Two of these studies used ovarian tissue as normal, one study used OSE as normal, and the fourth used peritoneal tissue as normal. In addition, four studies were also identified that compared metastasis to primary tumors. These were Adib et al. [42], Tothill et al. [46], Anglesio et al. [47], and Bittner (unpublished). In both cases, expression of genes related to TGFβ superfamily signaling (ligands and receptors) was analyzed.

Kaplan–Meier analysis

OvMark (http://glados.ucd.ie/OvMark/index.html) [48] was used to generate Kaplan–Meier curves for genes of interest. Specifically, OvMark was used to analyze disease-free survival (DFS) in 650–1950 patients (dependent on the gene(s) analyzed) across 14 GEO datasets. While the program does not allow the exclusion of low-grade serous tumors, >2/3 of serous tumors in the datasets are high-grade [48]. Additionally, all available serous samples were used, regardless of therapy, residual tumor status, grade, or FIGO stage, in order to maximize the number of patients included in the analysis.

Copy number and mRNA analysis in cBioPortal

cBioPortal (http://www.cbioportal.org/index.do) was used to analyze copy number and mRNA expression for components of activin A signaling in the TCGA provisional dataset. All complete tumors (n = 182) were analyzed for *ACVR1B*, *ACVR2A*, and *INHBA* expression. Samples were analyzed for amplification of INHBA, ACVR1B, and ACVR2A in the genome or altered mRNA expression was defined as a Z score less than −2 or greater than 2.

RT-PCR

RNA was isolated from OVCAR4 cells with TRIzol (15596026, ThermoFisher, Waltham, MA) per the manufacturer's instructions. RNA was treated with DNase I for 20 min at 37 °C and reverse transcribed with the iScript Reverse Transcription Supermix kit (170-8841, Bio-Rad, Hercules, CA). Each PCR reaction contained 25 ng OVCAR4 cDNA, 1× Taq Reaction Buffer, 1.25 U Taq, 200 μM dNTPs, 0.8 μM forward and reverse primers in a final volume of 25 μl. PCR conditions consisted of an initial denaturation step at 95 °C for 30 s followed by 25 cycles of 95 °C for 30 s, 52 °C for 60 s, and 68 °C for 30 s. The final extension was 68 °C for 60 s. Products were separated on a 2% agarose cell containing ethidium bromide and images were captured with a FluorChem E (ProteinSimple, San Jose, CA).

Statistical analysis

Each experiment was replicated at least three times and data is presented as mean ± SEM. Data were analyzed by a T-test, paired T-test, or ANOVA followed by Dunnett's post hoc analysis. $P \leq 0.05$ was considered significant. Analysis was carried out with PRISM version 6.0b.

Results

Activin A stimulates migration of the FTE

The predilection for HGSOC originating from the FTE to colonize the ovary [5,10] suggests that the ovary may secrete chemotaxic molecules. Activin A, a member of the TGFβ superfamily, is a major ovarian protein hormone produced by large follicles and the corpus luteum (CL). Activin A is well known to stimulate migration in other cancers [28]. Therefore, the ability for MOE (murine oviductal epithelial) cells to respond to activin A and TGFβ1 (as a member of the same superfamily) was tested. Activin A (10 ng/ml) induced an 8-fold increase in phospho-Smad2/3 levels in MOE cells at 60 min ($P < 0.0001$), and these levels remained significantly increased (∼ 5-fold) at 120 min (Fig. 1A and B). TGFβ1 (10 ng/ml) increased phospho-Smad2/3 levels to a greater extent than activin A, reaching a ∼ 25-fold increase at 30 and 60 min ($P < 0.05$), before returning to baseline by 120 min (Fig. 1C and D). To determine if activin A and TGFβ1 induce protein expression changes consistent with migration in MOE cells, E-cadherin and vimentin levels were measured in response to 24 h of treatment. Both activin A and TGFβ1 (10 ng/ml) resulted in a 65% reduction in E-cadherin expression ($P < 0.05$). They also increased vimentin expression ($P < 0.05$), though activin A increased vimentin levels to a greater extent than TGFβ (44% vs 25%, Fig. 1E–G).

As measured using a scratch assay, 10 ng/ml of activin A increased migration of MOE cells by 90% ($P < 0.05$), with higher concentrations (20 and 40 ng/ml) having approximately the same effect (90% and 107%, respectively; Fig. 1H; $P < 0.05$). Surprisingly, TGFβ1 had no effect on migration at any concentration tested (5–40 ng/ml; Fig. 1I). In agreement, 50 ng/ml of activin A increased migration in a Boyden chamber assay 50% over control ($P < 0.05$), and TGFβ1 had no effect (Fig. 1J and K). Next, the effect of activin A on proliferation was explored. Activin A was unable to stimulate proliferation in MOE cells cultured in serum-free media. Activin A was also unable to inhibit proliferation of MOE cells in the presence of 10% FBS (Fig. S1), which agrees with our previous study showing that TGFβ1 or SB431542 (activin and TGFβ inhibitor) had no effect on proliferation of murine FTE in a 3D culture system [49]. These results suggest that the TGFβ superfamily does not similarly regulate proliferation of the FTE and OSE [50]. These results also highlight a unique role for activin A to drive migration of the FTE, and possibly metastasis to the ovary.

Activin A stimulates migration through an AKT/MEK/RAC1 dependent pathway

Next, the pathway by which activin A stimulates migration was explored. The finding that TGFβ1 increased phospho-Smad2/3 levels, but did not increase migration, suggested that the increased migration was Smad independent. Confirming this, a previously validated dominant negative Smad2 (Smad2DN) construct [37,38] did not abrogate the migratory effect of activin A (Fig. 2A). The Smad2DN construct blocked phosphorylation of Smad2/3 (Fig. 2B). Therefore, activin A stimulated migration of MOE cells through Smad-independent signaling pathways.

TGFβ1 superfamily members can activate signaling pathways that do not rely on Smad2/3 phosphorylation in a ligand- and cell-dependent manner [17]. Therefore, the ability of activin A and TGFβ1 to stimulate PI3K/AKT and MEK/ERK signaling was evaluated via western blot. Activin A increased phospho-ERK levels by 8.5-fold at 30 min ($P < 0.05$, Fig. 2C and D). Activin A resulted in a

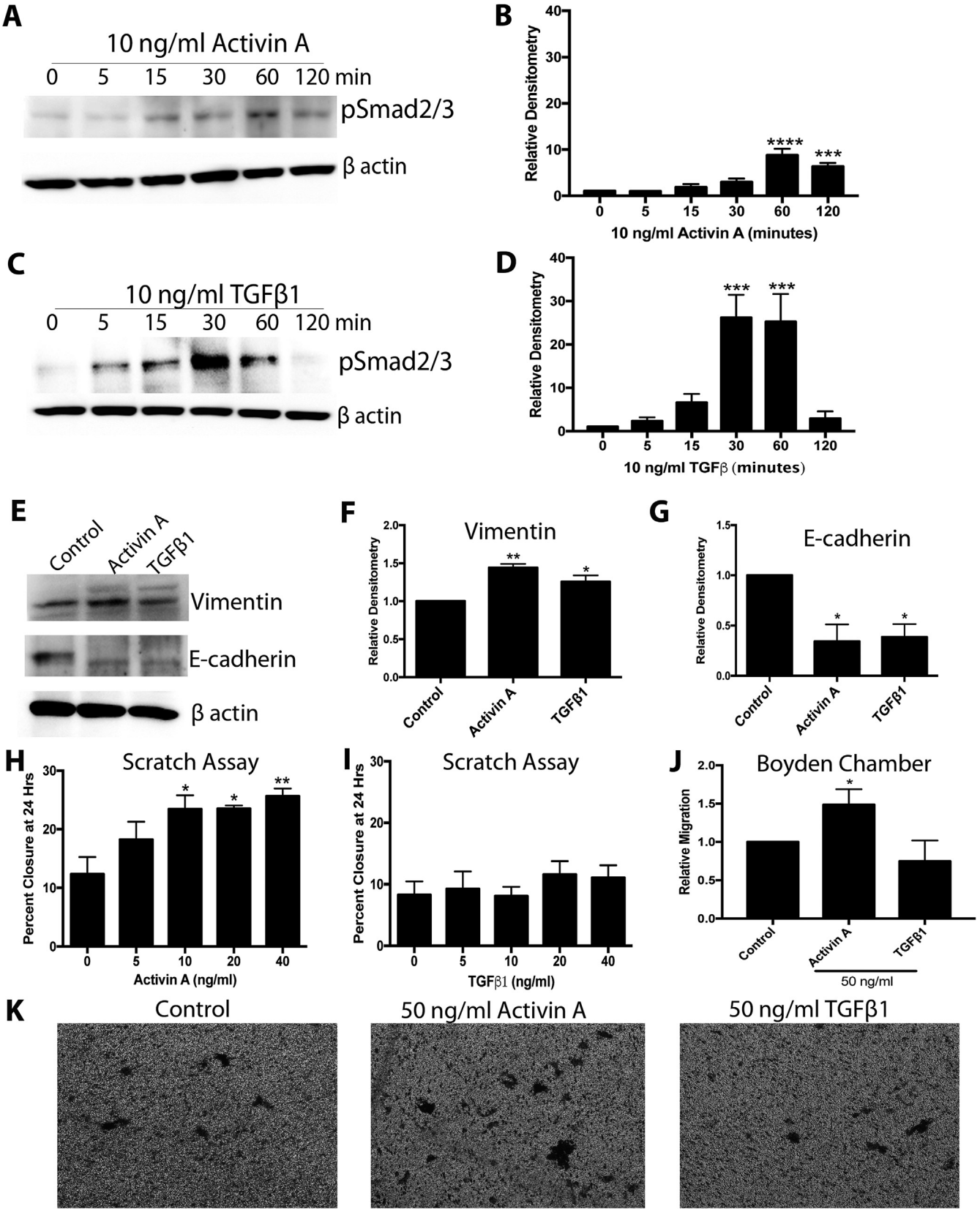


Fig. 1. Activin A and TGFβ1 alter expression of EMT markers in MOE cells, but only activin A stimulates migration. A) Representative western blots for phospho-Smad2/3 in MOE cells treated with activin A. B) Densitometry for phospho-Smad2/3 in response to activin A. C) Representative western blots for phospho-Smad2/3 in response to TGFβ1. D) Densitometry data for phospho-Smad2/3 in MOE cells treated with TGFβ1. E) Representative westerns for MOE cells treated with activin A or TGFβ1 for 24 h and probed for vimentin and E-cadherin. F and G) Band densitometry for vimentin and E-cadherin. H) and I) Scratch assay for MOE cells treated with 0–40 ng/ml activin A or TGFβ1. J) Relative migration of MOE cells in response to 50 ng/ml activin A or TGFβ1 in a Boyden chamber. K) Representative images of migrated cells in a Boyden chamber. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n ≤ 3.

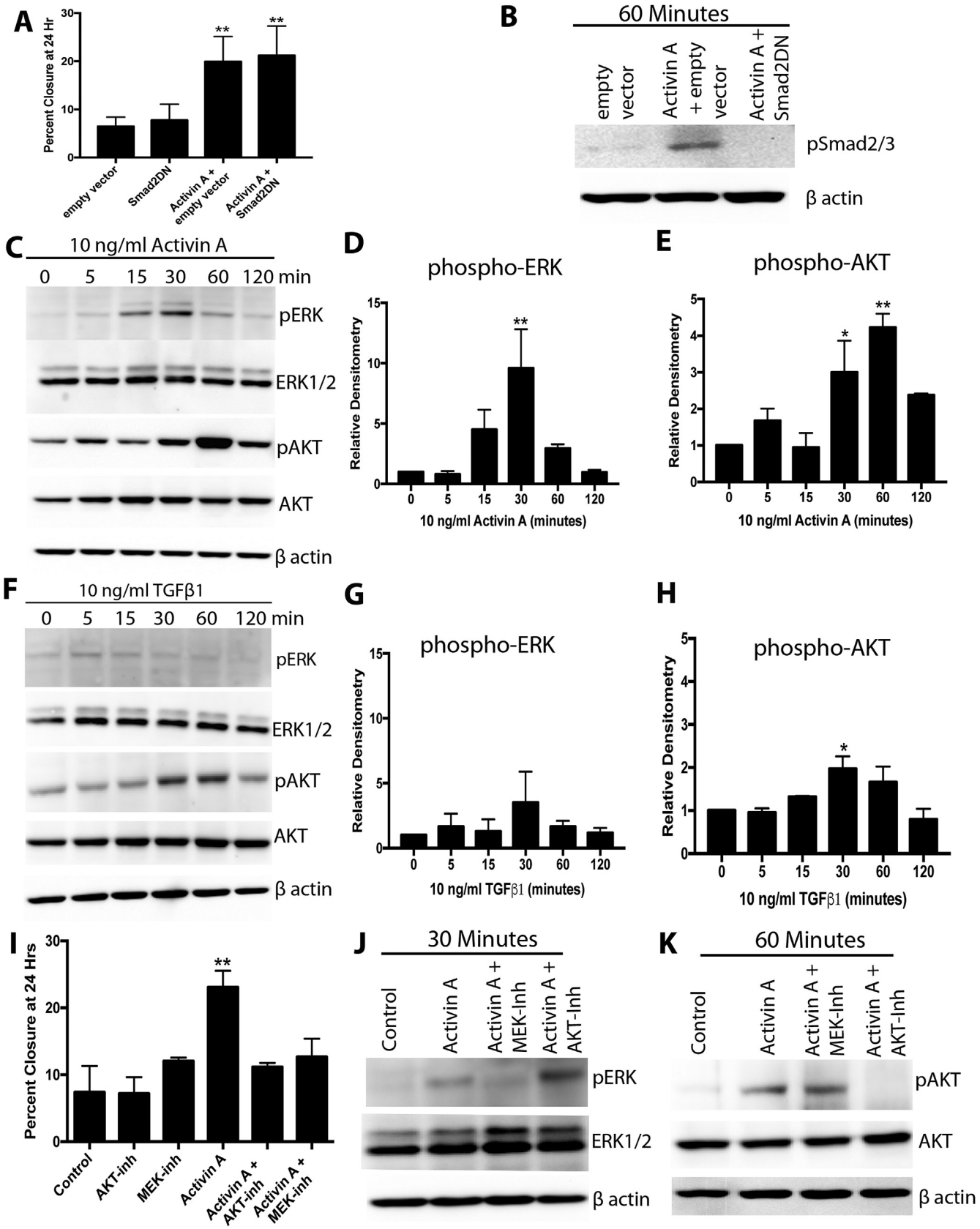


Fig. 2. PI3K/AKT and MEK/ERK but not Smad2/3 pathways are required for activin A-stimulated migration of MOE cells. A) Migration of MOE cells transfected with a dominant negative Smad2 (Smad2DN) construct, empty vector, and treated with 10 ng/ml activin A as indicated. B) Western blot for phospho-Smad2/3 in MOE cells treated activin A and Smad2DN construct as indicated. C-E) Example western blots (C) and densitometry for phospho-ERK (D) and phospho-AKT (E) of MOE cells treated with 10 ng/ml activin A. F-H) Example western blot (F) and densitometry of phospho-ERK (G) and phospho-AKT (H) of MOE cells treated with 10 ng/ml TGFβ1. I) Migration of MOE cells treated with activin A (10 ng/ml), an AKT inhibitor (MK2206), or a MEK inhibitor (U0126) as indicated. J and K) Example western blots for phospho-AKT and phospho-ERK in MOE cells treated with activin A for 30 (J) or 60 (K) minutes in the presence of an AKT inhibitor (MK2206) or a MEK inhibitor (U0126). *$P < 0.05$, **$P < 0.01$, $n \leq 3$.

~3-fold increase in phospho-AKT levels after 30 min ($P < 0.05$), and phospho-AKT was 4.2-fold higher at 60 min relative to 0 min ($P < 0.01$; Fig. 2C and E). In contrast, TGFβ1 had no effect on phospho-ERK levels at any time point examined (Fig. 2F and G). TGFβ1 increased phospho-AKT levels, but to a lesser extent than activin A. Phospho-AKT levels were only ~2-fold higher at 30 min with TGFβ1 treatment ($P < 0.05$; Fig. 2F and H). To confirm that these pathways were required for activin A-induced migration, scratch assays were performed in the presence of MK2206 (AKT inhibitor) and U0126 (MEK inhibitor). Activin A alone significantly increased migration. Both inhibitors completely abolished activin A-stimulated migration, bringing migration values back to control levels (Fig. 2I). Confirming that these inhibitors are specific in MOE cells, MK2206 completely blocked activin A-stimulated AKT phosphorylation, but not ERK phosphorylation. U0126 inhibited activin stimulated ERK phosphorylation, but not AKT (Fig. 2J and K). These results suggested that activin A stimulated phospho-AKT and phospho-ERK in parallel of each other, and indicate both pathways are required to stimulate migration of the FTE.

Activin A and TGFβ1 have been shown to activate members of the Rho GTPase family (mainly RhoA, Rac1, and Cdc42), which are major regulators of the Apr2/3 complex and cell migration [51]. Therefore, the ability of each of these proteins to mediate activin A-induced migration was tested using Rhosin (RhoA inhibitor), NSC23766 (Rac1 inhibitor), and MLS573151 (Cdc42 inhibitor). By themselves, none of the inhibitors changed migration. Activin A increased migration of MOE cells approximately 60% in the presence of Rhosin and MLS573151, indicating that RhoA and Cdc42 are not the primary mediators of activin A-induced migration. In contrast, NSC23766 completely abrogated migration (Fig. 3A), suggesting a role for Rac1. To confirm a role for Rac1 in migration, the effect of a $Rac1^{siRNA}$ was evaluated. In the presence of luciferase siRNA, activin A increased migration 175% ($P < 0.05$). In contrast, $Rac1^{siRNA}$ completely blocked the migratory effect of activin A (Fig. 3B). These results demonstrate that Rac1 is necessary to mediate activin A-induced migration.

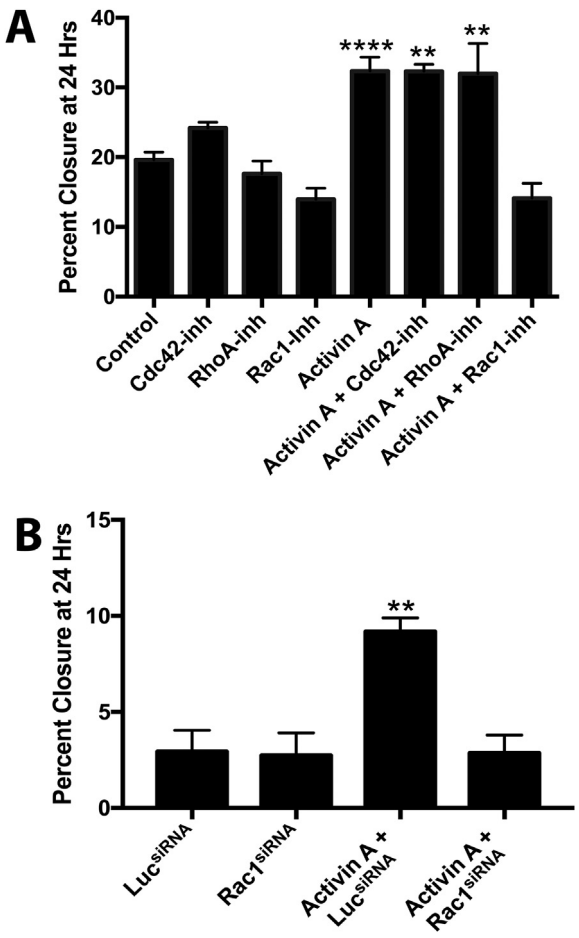


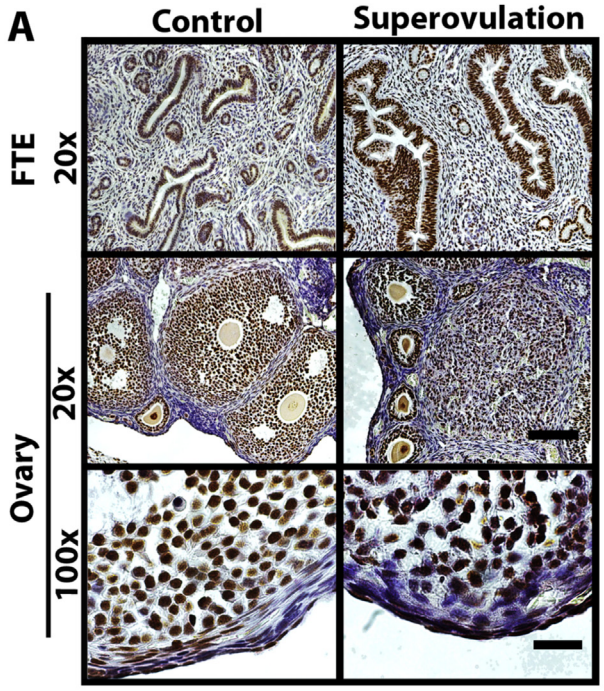
Fig. 3. Rac1 mediates activin A-induced migration. A) Migration of MOE cells in response to activin A, Cdc42 inhibitor (LS573151), RhoA inhibitor (Rhosin), or Rac1 inhibitor (NSC23766) as indicated. B) Migration in MOE cells treated with activin A, and luciferase siRNA, or Rac1 siRNA. **$P < 0.01$, ****$P < 0.0001$, $n \leq 3$.

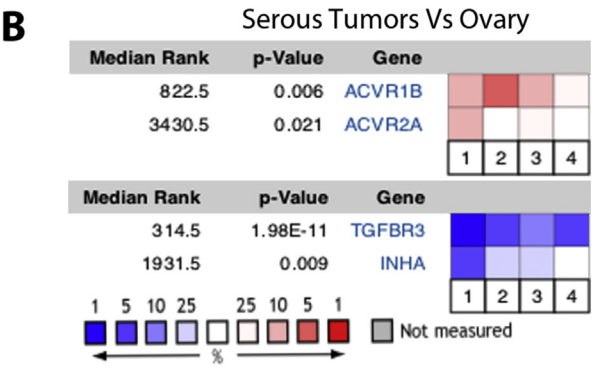
The FTE responds to ovarian activin A in vivo

Superovulation was used to determine if ovarian factors (activin A or TGFβ) could activate receptors in the FTE (called oviducts in mice) of CD1 mice. Mice were treated with vehicle (control) or PMSG and hCG to induce superovulation, and then the ovary and fallopian tubes were evaluated via immunohistochemistry. In control mice, there was noticeable phospho-Smad2/3 immunostaining in the FTE, and superovulation increased the intensity of this immunostaining. Similarly, high-magnification of the OSE revealed Smad2/3 staining in control mice, which was increased in response to superovulation (Fig. 4A).

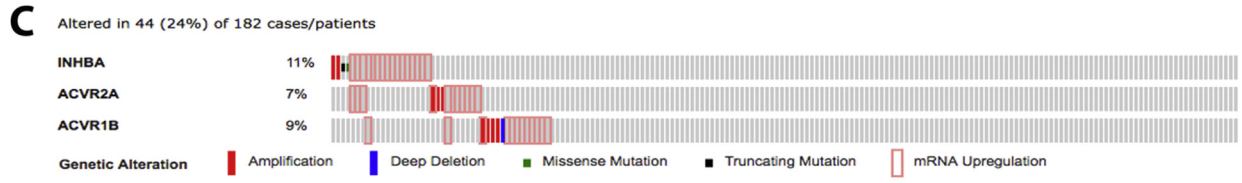
In order to address whether human ovarian tumor samples express mRNA that supports a role for activin A in ovarian spread, the TGFβ superfamily was evaluated in HGSOCs compared to normal ovaries with Oncomine™. Four studies in Oncomine™ were identified that compared serous tumors to normal ovarian tissue [42–45]. In these 4 datasets combined, both the type I and type II activin receptors (*ACVR1B* and *ACVR2A*) were significantly overexpressed in serous tumors relative to the normal ovary (Fig. 4B; $P < 0.05$). Interestingly, *ACVR2B*, which can function as an activin or nodal receptor, was overexpressed in 2 of 4 studies, but this did not reach statistical significance ($P = 0.33$). No other receptors (*TGFBR1*, *TGFBR2*, *ACVR1*, and *ACVR1C*) or ligands (*INHBA*, *INHBB*, *TGFB1*, *TGFB2*, *TGFB3*, *NODAL*) were overexpressed in serous tumors relative to normal ovaries (Fig. S2). Under-expression analysis revealed that *INHA* and *TGFB3* (also known as betaglycan), both of which reduce activin signaling, were significantly lower in serous tumors ($P < 0.05$, Fig. 4B). In addition, *TGFBR2* and *TGFB2* were significantly lower ($P < 0.05$, Fig. S2) in serous tumors. *TGFBR1* and *NODAL* tended to be lower in serous tumors relative to normal ovaries but this did not reach statistical significance ($P < 0.01$, Fig. S2). Four studies were also identified that compared distant metastatic tumors to tumors located in the ovary. Confirming a role for activin A in migration of HGSOC to the ovary, but not necessarily all metastatic sites, tumors that had metastasized away from the ovary significantly over-expressed *INHBA* and under-expressed *ACVR1B* (Fig. S2). These changes would make tumors less sensitive to ovarian activin. Probing the TCGA dataset using cBioPortal revealed that *INBHA* was altered in 11% of cases, *ACVR2A* in 7%, and *ACVR1B* in 9%. Overall, *INHB, ACVR2A, or ACVR1B* was altered in 24% of cases, with almost all alterations being amplification of the gene or up-regulation at the mRNA level (Fig. 4C). These results support the hypothesis that activin A may encourage ovarian colonization of tumors originating in the FTE.

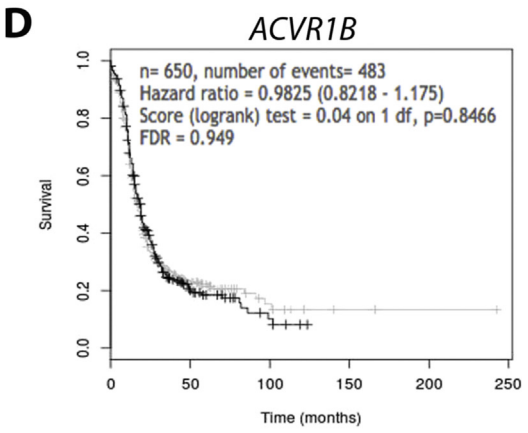
OvMark [48] was used to generate Kaplan–Meier plots for patients with high (above the median) or low (below the median) expression of genes involved with activin A and TGFβ signaling. While expression of *ACVR1B* had no effect on disease-free survival (DFS) in patients (Fig. 4D), patients with high expression of *ACVR2A* (Fig. 4E) and *INHBA* (Fig. 4F) experienced significantly shorter DFS than patients with low expression (HR = 1.39, FDR adjusted P value = 0.031 and HR = 1.266, FDR adjusted P value = 0.030, respectively). Interestingly, combining *ACVR2A* and *INHBA* resulted

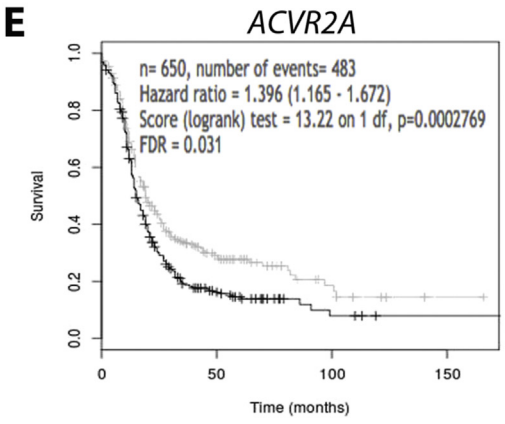


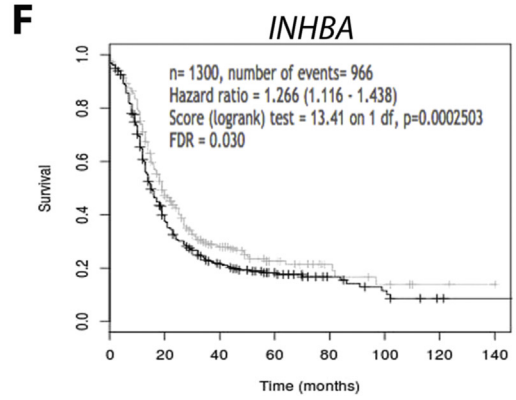


Study
1.Ovarian Serous Adenocarcinoma vs Normal
Adib Ovarian, Br J Cancer, 2004
2. Ovarian Serous Adendocarinoma vs Normal
Hendrix Ovarian, Cancer Res, 2006
3. Ovarian Serous Adenocarcinoma vs Normal
Lu Ovarian, Clin Cancer Res, 2004
4. Ovarian Serous Adenocarcinoma vs Normal
Yoshihara Ovarian, Cancer Sci, 2009









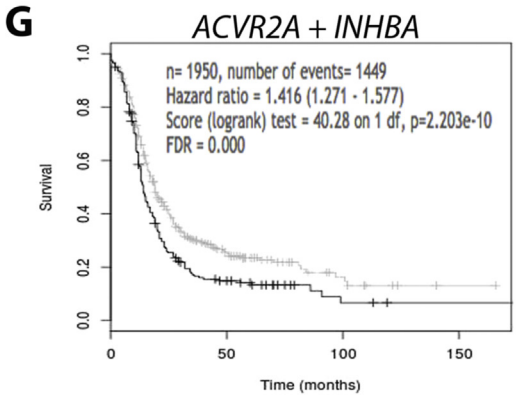


Fig. 4. The fallopian tube epithelium responds to ovarian activin *in vivo* and is correlated with disease-free survival (DFS). A) Immunostaining for phospho-Smad2/3 in the fallopian tube and ovary of control and superovulated mice. Scale bar = 50 μm at 20× and 10 μm at 100×. B) Oncomine analysis for *ACVR1B*, *ACVR2A*, *TGFBR3*, and *INHA* in serous tumors relative to normal ovary across 4 studies as indicated. Top: overexpressed genes (red); Bottom: under-expressed genes (blue). C) Percentage of tumors with altered *INHBA*, *ACVR2A*, and *ACVR1B* in TCGA using cBioPortal. D-G) Disease Free Survival (DFS) Kaplan–Meier plots from OvMark for *ACVR1B* (D) and *ACVR2A* (E), *INHBA* (F), and *ACVR2* + *INHBA* (G) in serous ovarian cancer patients.

in the biggest difference between high-expression and low-expression groups (Fig. 4G; HR = 1.416 and FDR adjusted P value < 0.001), suggesting that an autocrine feedback loop with activin A may facilitate spread of serous tumors. High expression of TGFβ (*TGFB1*, *TGFB2*, or *TGFB3*) or TGFβ receptors (*TGFBR1* and *TGFBR2*) was not associated with any difference in DFS (Fig. S3). These results suggest a unique role for activin A in the progression of HGSOC.

Activin A stimulates migration in HGSOC cell lines

Next, the ability of activin A to drive migration of tumorigenic cells was evaluated using OVCAR3 and OVCAR4 cells, which are likely to represent HGSOC [36,52]. Activin A induced a dose-dependent increase in migration in OVCAR3 cells. Activin A at 20 and 40 ng/ml significantly increased migration ($P < 0.01$) over control (Fig. 5A). Activin A resulted in increased levels of phospho-AKT and phospho-ERK in OVCAR3 cells (Fig. 5B). U0126 (ERK inhibitor), but not MK2206 (AKT inhibitor), significantly reduced basal migration in OVCAR3 cells. Activin A (20 ng/ml) significantly increased migration, which was completely abrogated by MK2206. U0126 blocked activin A-induced migration, but it also reduced migration to less than control ($P < 0.05$, Fig. 5C). NSC23766 (Rac1 inhibitor) completely blocked activin A-induced migration (Fig. 5D). These data confirm that activin A stimulates migration of HGSOC in an AKT/ERK/Rac1-dependent pathway in OVCAR3.

In OVCAR4 cells, activin A had less of an effect of migration with only the highest concentration of activin A (40 ng/ml) having a slight (45%), but significant, enhancement of migration ($P = 0.026$; Fig. 6A). The effect of FBS on migration of MOE cells and OVCAR4 cells (which migrate at approximately the same rate) was determined. FBS (10%) increased migration of MOE cells by 400% ($P < 0.0001$). In contrast, 10% FBS increased migration of OVCAR4 cells by only 52% ($P < 0.05$; Fig. 6B), indicating that OVCAR4 cells are less reliant on exogenous stimulation to induce migration. RT-PCR revealed that OVCAR4 cells produce *INHBA* and are capable of making activin A, while *TGFB1*, *TGFB2*, *TGFB3*, *INHBB*, and *Nodal* were undetectable (Fig. 6C). In serum-free media, SB431542 (inhibitor of TGFβ, activin A, and nodal) reduced migration of OVCAR4 cells by 35% ($P < 0.01$), and follistatin (a specific activin inhibitor) reduced migration 20% ($P < 0.05$; Fig. 6D). Inhibition of AKT with MK2206 significantly reduced migration of OVCAR4 cells by 35%. Surprisingly, the MEK inhibitor (U0126) had no effect on migration of this cell line (Fig. 6E). NSC23766 also inhibited migration (38%, $P < 0.05$), confirming a role for Rac1. These results show that while some cell lines (MOE and OVCAR3) are dependent on exogenous activin A to stimulate migration, other cancer cell lines (OVCAR4) produce activin A to stimulate their own migration.

Discussion

The current model of ovarian cancer metastasis to the omentum is that tumorigenic cells are shed by the ovarian tumor, reach the omentum through passive flow of peritoneal fluid, and invade at milky spots, using energy stored in adjacent adipocytes [53,54]. However, much less is known about why tumors that start in the fallopian tube epithelium migrate to the ovary. It is clear that ovulation increases a woman's risk of developing ovarian cancer [55]. However, the mechanism(s) by which ovulation increases the risk of ovarian cancer is less clear. This is important because recent evidence indicates that colonization of the ovary by FTE-derived tumors may be an important step in the progression of HGSOC [10,13] and suggests that this step may represent a therapeutic target in women at high risk of developing HGSOC [10]. The ovary produces a large number of hormones and growth factors that may stimulate migration of tumorigenic FTE cells to the ovaries.

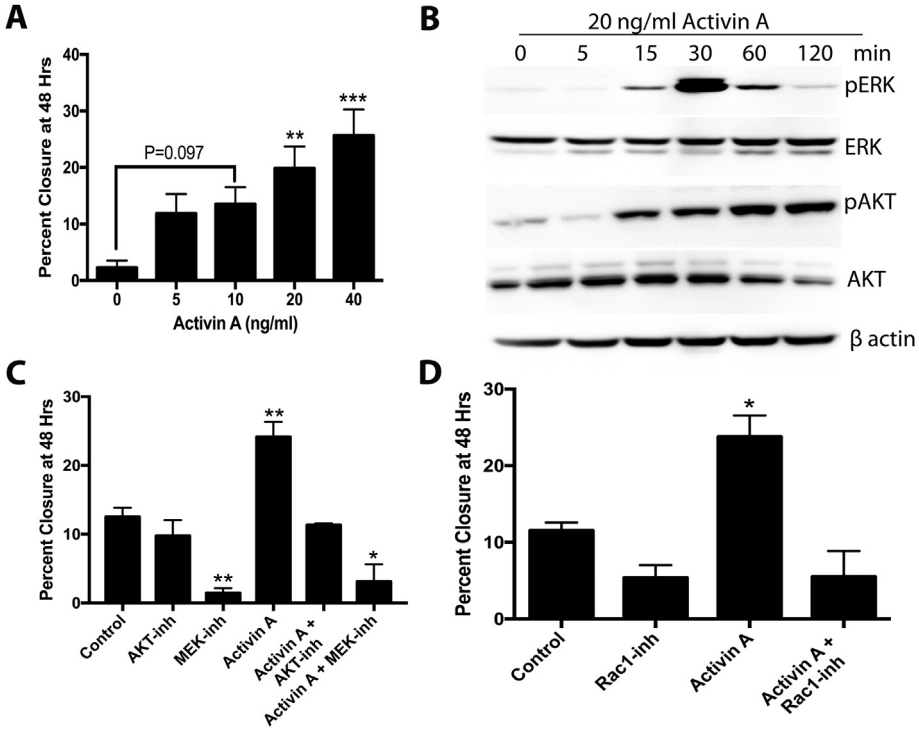


Fig. 5. Activin A stimulates migration of OVCAR3 via AKT/MEK/RAC1. A) Migration of OVCAR3 cells in response to activin A (0–40 ng/ml). B) Example western blot for pAKT, AKT, pERK, ERK, and β actin in response to 20 ng/ml activin A over 2 hr. C) Migration in OVCAR3 in response to activin A, an AKT inhibitor (MK2206) and a MEK inhibitor (U0126) as indicated. D) Migration of OVCAR3 cells treated with activin A or a Rac1 inhibitor (NSC23766) as indicated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $n \leq 3$.

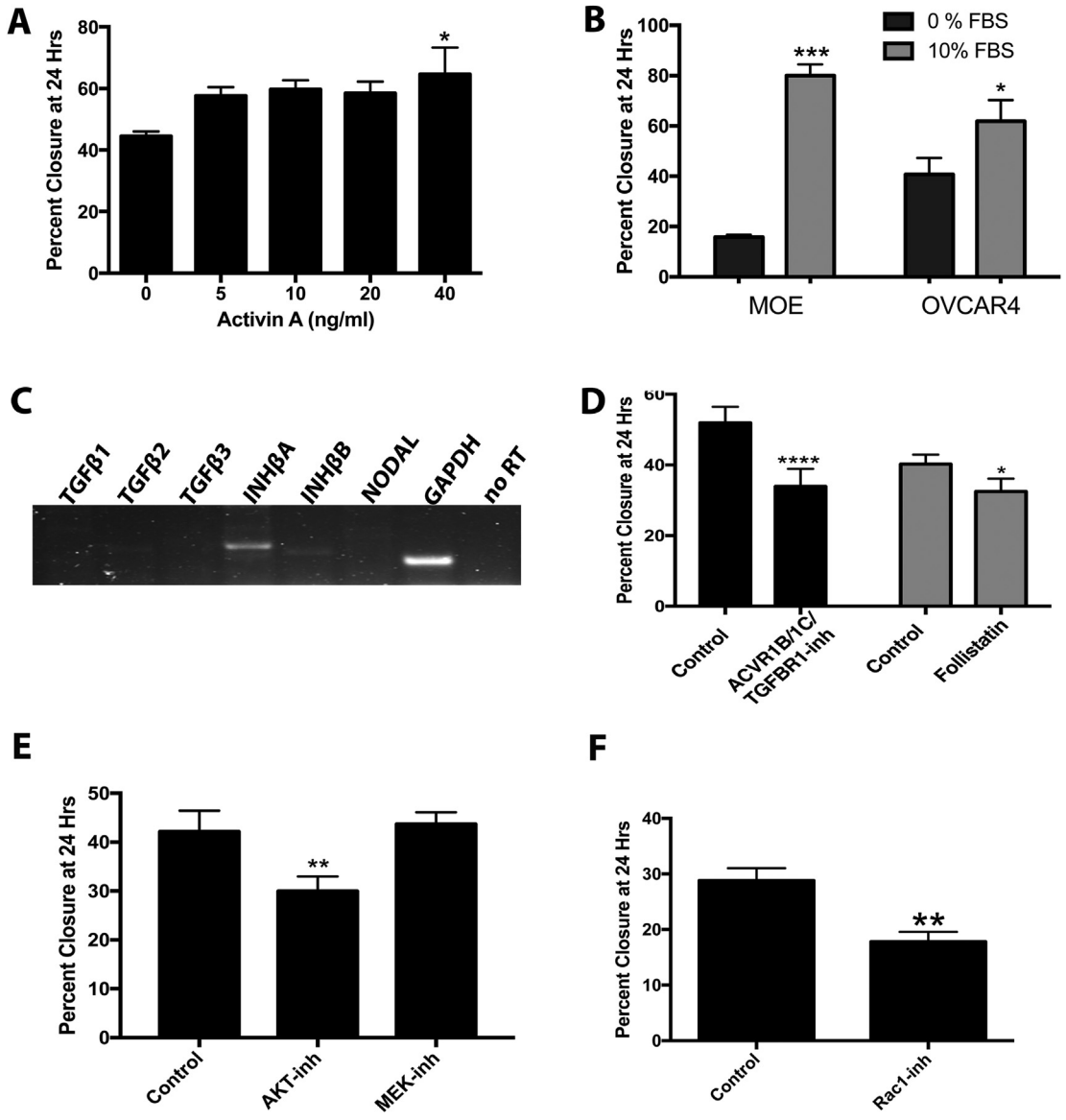


Fig. 6. Activin A stimulates migration of OVCAR4 cells in an autocrine manner. A) Migration of OVCAR4 cells in response to activin A (0–40 ng/ml). B) Migration of MOE and OVCAR4 cells in response to 10% FBS. C) RT-PCR bands showing OVCAR4 cells express *INHBA* mRNA but not *TGFB1*, *TGFB1*, *TGFB3*, or *NODAL*. D) Migration of OVCAR4 cells treated with an ACVR1B, ACVR1C and TGFBR1 inhibitor (SB431542) or a specific activin inhibitor (follistatin). E) Migration in OVCAR4 cells treated with an AKT inhibitor (MK2206) or a MEK inhibitor (U0126). F) Migration in OVCAR4 cells treated with a Rac1 inhibitor (NSC23766). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n ≤ 3.

Estradiol is a major ovarian hormone, but it had no effect on migration of MOE cells [39]. In addition, steroids circulate systemically, making it unlikely that they stimulate the local migratory step from the fallopian tube to the ovary. More likely, a set of locally acting proteins from the ovary stimulates migration of tumorigenic cells. Yang-Hartwich et al. showed that stromal differentiation factor 1 (SDF-1) stimulated migration of OTIC cells [15]; however, OTICs cells have an unknown cell of origin, and therefore may not represent the FTE.

Superovulation increased phospho-Smad2/3 levels in the OSE and FTE. It is unclear if this increase was due to activin A or TGFβ1. Unfortunately, small molecule inhibitors (e.g. SB431542 or LY2109761) of the activin receptor also inhibit TGFβ receptors, making such a distinction difficult *in vivo*. However, reported concentrations of activin A in follicular fluid are higher than those reported for TGFβ [30,56], suggesting that activin A may play a role. More importantly, these results show that ovarian hormones reach the FTE in sufficient concentrations to activate downstream signaling pathways. Mice have an ovarian bursa which may result in a closer proximity between the ovary and FTE than would occur in humans; however, phospho-Smad2/3 has also been detected in the human FTE [57].

Here we show that activin A increases migration of MOE and OVCAR3 cells, which agrees with effects of activin A in other cancer types [28]. Activin A also resulted in increased EMT, evidenced by significantly higher expression of vimentin and lower expression of E-cadherin in MOE cells. In agreement, Basu et al. found that activin A increased mRNA expression of *ZEB1*, *ZEB2*, *SNAI1* and *SNAI2* in OAW-42 cells [32]. SB-431542 (an activin and TGFβ inhibitor) reduced *SNAI1*, *TWIST1*, and *ZEB2* expression in spheroids grown from primary ovarian cancer ascites cells [58].

Interestingly, a woman's risk of ovarian cancer increases after menopause. At the same time, there is a clear link between the number of ovulations and ovarian cancer risk [59]. One potential

mechanism to explain this apparent paradox is that tumorigenic cells colonize the ovary prior to menopause, but tumors are not diagnosed until after menopause. In support of this hypothesis, a recent mathematical analysis calculated the latency period for ovarian cancer to be 44 years [60]. The mechanisms resulting in tumor metastasis beyond the ovary are poorly understood and are likely complex. One possibility is that tumors may become resistant to ovarian activin A prior to distant metastasis. In support of this, Oncomine data identified that distant tumors had higher levels of *INHBA* and lower *ACVR2A*, both of which would result in decreased sensitivity to activin A from the ovary. In agreement, OVCAR4 apparently stimulated their own migration via activin A secretion. Also, activin A concentrations are higher in postmenopausal women with epithelial ovarian cancer [61], and activin A levels have been shown to correlate with disease reoccurrence [62].

The omentum, a frequent site of colonization in HGSOC, also produces activin A [63]. It is unknown if ovarian cancer associated fibroblasts (CAFs) produce activin A. In colon cancer cells, CAFs enhanced transcription of TGFβ [64]. Tumor-associated myofibroblasts from the tongue produced increased amounts of activin A, which drove proliferation of oral squamous cell carcinoma cells [65]. Thus, activin A may play a role in spread of HGSOC beyond the ovary considering that it is produced by multiple sources including HGSOCs and CAFs. If further validated, activin A signaling may be an attractive target to prevent the spread of HGSOC, especially given the recent development of a small molecule activin A antagonist [66] and the ongoing phase I clinical trial using an ACVR2B soluble receptor ligand trap in patients with ovarian, fallopian tube, or endometrial cancers (ClinicalTrails.gov Identifier: NCT02262455). Importantly, these strategies may be more successful if used in patients when disease is still confined to the fallopian tube.

In the current study, activin A had no effect on proliferation of MOE cells. In agreement, neither TGFβ1 nor SB431542 had any effect on proliferation in a 3D model of the mouse FTE [49]. In contrast, activin A and TGFβ have repeatedly been shown to inhibit proliferation of OSE cells [49,50], and *in vivo* deletion of Smad3 resulted in increased proliferation of OSE [67]. The role of activin A and TGFβ in ovarian cancer cell lines is mixed. Steller et al. found that activin A had no effect on proliferation of OVCA429, HEY, and A2780-cp cells and increased proliferation of OCC1, SKOV3, OVCAR3, and A2780-S cells [34]. In contrast, Ramachandran et al. found that activin A inhibited proliferation of OVCAR4, NZOV2, NZOV10, NZOV5, and OVCAR3 cells, but had no effect on proliferation of COV644, OVCAR5, SKOV3, NZOV4, NZOV9, NZOV11, and NZOV13 [68]. Based on the results presented here, it is possible that the mixed response of ovarian cancer cell lines reflects their tissue of origin. It is also possible that the response of cells is dependent on changes that occur during transformation. For example, proliferation in response to TGFβ has been linked to p53 signaling [49], and p53 is mutated in almost all cases of HGSOC [69].

In conclusion, activin A (but not TGFβ1) stimulated migration of FTE and HGSOC cells through phospho-AKT and phospho-ERK signaling. In patients, serous ovarian tumors had higher levels of *AVCR1B* and *ACVR2A* compared to normal ovaries, while *INHA* and *TGFB3* transcripts were lower. Distant ovarian tumors had higher levels of *INHBA* and *ACVR2A* relative to tumors localized to the ovary. High expression of both *INHBA* and *ACVR2A* was associated with shorter DFS in serous cancer patients, and *INHBA*, *ACVR1B*, and *ACVR2B* were frequently altered in HGSOC patients. These data indicate that activin A plays an important role in the early pathogenesis of HGSOC, and may contribute to the spread of FTE-derived tumors to the ovary. An increased understanding of the role of ovarian factors in the colonization of the ovary may uncover therapeutic targets for FTE-derived HGSOC.

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Conflict of interest statement

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2017.01.011.

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